By enhancing the genetic code it is possible to incorporate unnatural amino acids (UAA) with new properties into proteins without restrictions on the site to incorporate the UAA. By using the Amber suppression method in Xenopus oocytes we have obtained high expression levels with the heterologously expressed voltage-gated Shaker potassium channel (Kv) harboring the fluorescent unnatural amino acid Anap at various key regions. Anap is environmentally sensitive which makes it capable of probing local conformational changes in the channel. In contrast to the traditional post-translational fluorophore labeling technique with thiol-chemistry, we are now capable of probing dynamics on the cytosolic side aswell as within the membrane bilayer. This opens a wide field of structural questions to be addressed since the important dynamics usually resides inside the cell or within the membrane. Anap was thus incorporated on each side of the S4 voltage sensor as well as on the S6 cytosolic gate. With voltage-clamp fluorometry we were able to determine that the four voltage sensors activate independently while the pore opening occurs cooperatively. Against previous models, pore opening required two cooperative movements. We also simultaneously labeled both ends of the voltage sensor S4 and found that dynamics of N- and C-termini differ. The successful use of fluorescent UAAs combined with voltage-clamp fluorometry has made it possible to study internal dynamics in electrogenic membrane proteins and will find widespread application in structural biology.

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Offsetting the Electric Field Sensed by K_V Channels through Residue Substitutions on Top of S1

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Kv channel subunits consist of 6 transmembrane segments (S1-S6) whereby the S1 through S4 segments assemble into a voltage sensing domain (VSD) that detects the membrane electric field. The positively charged S4 segment forms the main component of the VSD and undergoes the largest reorientations upon a membrane de- or hyperpolarization, generating a transient gating current. The S1-S3 segments surround the S4 and facilitate the latter's movement across the hydrophobic plasma-membrane. A positive (lysine) and negative (aspartate) charge substitution scan at the extracellular end of the S1 segment in the *Shaker*-type Kv1.5 channel indicated that this region is sufficiently close to the S4 segment such that it modulates the local membrane electric field. At positions E268, E272, F273 and E276 a charge substitution or charge introduction exerted a surface charge effect and shifted the voltage dependence of channel opening accordingly. Surprisingly, these residues, which modulated the electric field, did not face the S4 in a predicted 3D structure of the Kv1.5 channel in the open state (homology model based on the crystal structure of the Kv2.1/Kv1.2 chimera). This suggests that the introduced charges affect the electrical field around the S4 segment in the closed state only. In conclusion, residues at the top of the S1 segment can state-dependently offset (polarize) the electric field sensed by the S4 segment, supporting that both segments are in close proximity. (This research was supported by fellowship CONACyT #203936 to EMM & grant FWO-G.0449.11N to DJS.)

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Allosteric Coupling of the Inner Activation Gate to the Outer Pore of a Potassium Channel

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In potassium channels, functional coupling of the inner and outer pore gates may result from energetic interactions between residues and conformational rearrangements that occur along a structural path between them. Here, we show that conservative mutations of a residue near the inner activation gate of the *Shaker* potassium channel (I470) modify the rate of C-type inactivation at the outer pore, pointing to this residue as part of a pathway that couples inner gate opening to changes in outer pore structure and reduction of ion flow. Because they remain equally sensitive to rises in extracellular potassium, altered inactivation rates of the mutant channels are not secondary to modified binding of potassium to the outer pore. Conservative mutations of I470 also influence the interaction of the *Shaker* N-terminus with the inner gate, keeping it open and separately promoting re-orientation of the outer pore.

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Enhancement of C-Type Inactivation by External Ca^{2+} and La^{3+}

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The ShB ΔN mutant T449A undergoes C-type inactivation after depolarization within ~100 ms in external solutions containing 2-4 mM Ca^{2+} and 0 K^+. Raising external Ca^{2+} to 40 mM, reduces peak I_K for a single pulse to ~ 20%, and virtually eliminates I_K with steady pulsing at 0.1 Hz. The effect of $Ca^{2+}$is reversible. Raising external K^+ to 5-20 mM (in 40 mM Ca^{2+}) largely restores I_K. How is Ca^{2+} suppression and K^+ restoration of I_K explained? Ca^{2+} blocks inward I_K through an open channel strongly at negative voltages. We propose that Ca^{2+} competes with K^+ at $Site_0$ (just outside the selectivity filter) where K^+ is strongly favored, but Ca^{2+} occupancy is enhanced at negative voltages. Ca^{2+} occupancy of $Site_0$ strongly reduces K^+ access to and occupancy of $Site_1$ (outermost site of the filter), allowing repulsion among the carbonyls of $Site_1$ to dilate this site, causing C-type inactivation. La^{3+} has almost the same crystal radius as Ca^{2+}, and is much more potent in suppressing I_K: with 10 μM La^{3+} externally, I_K of T449A is largely eliminated (reversibly). We postulate that trivalent La^{3+} has high affinity for $Site_0$. Neither Ca^{2+} nor La^{3+} has strong effects on T449V, which does not exhibit C-type inactivation.

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Shaker Kv Channel's Sugar Remotion in Real-Time

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Voltage-gated K^+ channels (Kv) are tetramers, each subunit containing six transmembrane segments (S1-S6). These segments make two functionally and structurally independent domains: an ion conduction pore (S5,S6), and voltage-sensor domains (S1-S4). Like many transmembrane proteins, Kv channels undergo extensive posttranslational modifications, with a significant percentage of the mature protein mass being composed of glycan moieties. Shaker Kv channels are glycosylated at two asparagine residues (N259 and N263) located in the first extracellular loop. Mutating asparagine residues to glutamine abolishes glycosylation but does not prevent cell surface expression of functional channels. Using the same population of Shaker Kv channels, we studied the function and toxin binding before and after deglycosylation by PNGase F. Binding properties of Hanatoxin, GxTx and Agitoxin remained unaltered by removing glycosylation moieties, suggesting that sugars attached to the channels are not intimately interacting with regions of the voltage sensor domains and the pore where these toxins bind. We also studied ionic and gating currents, before and after deglycosylation with PNGase F. Opening kinetics were substantially slowed down by deglycosylation, as reported previously in many members of the Kv family. Total charge distribution mediated by the voltage sensors of the non-conductive W434F mutant Shaker channels shifted only ~3-4 mV towards positive potentials after deglycosylation. Overall our data provides direct proof to understand how glycosylation contributes to Kv channel function.

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Locking the Open State of a Voltage-Dependent Concatemer Potassium Channel with Metal Bridges

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Voltage-gated K^+ (K_v) channels are homotetramers. Each subunit is formed by six transmembrane segments, S1 to S6. The permeation pathway is formed by S5, the P-region and S6 from each subunit, that assemble around a central canal through which K^+ permeate. In response to voltage changes, the voltage sensor domain (S1-S4) undergoes conformational changes that are coupled to the opening and closing of an intracellular gate located at the inner end of S6. It is known, that a cysteine at position 476 in S6 (near the gate) traps the mutant channel in the open state when Cd^{2+} is added to the intracellular solution. This behavior is caused by a metal bridge between the cysteine at 476 of one subunit and a native histidine at 486 in an adjacent subunit. We constructed a Shaker K_v concatemer that has all 4 subunits linked at the DNA level. When all four V476 are mutated to cysteine, intracellular Cd^{2+}, locked the channels open as in wild-type channels. We are presently developing concatemer channels with one, two and three metal bridges to assess one by one their contribution to the opening of the channels. Because the metal bridges can be disrupted by a H486Y mutation, this approach should allow us to discern the assembly of a functional concatemer channel in the membrane.

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Mutations in the Cavity Affect the Rate of Slow Inactivation in Shaker K^+ Channels

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It has been reported earlier that the activation and inactivation gates of Shaker channels are coupled, and this coupling might be mediated by a rotational motion of the S6 helix. We hypothese that either the electrostatics or the volume/hydrophobicity of the side chains pointing into the cavity can affect this coupling. Accordingly, we have introduced side-chains at position 470 in a